

APPLICATION  
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TITLE: TYMPANIC MEMBRANE PATCH

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## TYMPANIC MEMBRANE PATCH

### CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Provisional Patent Application Serial No. 60/271,105, filed on February 23, 2001, which is incorporated herein by reference in its entirety.

### TECHNICAL FIELD

This invention relates to tissue engineering of a tympanic membrane patch.

### BACKGROUND

The treatment of recurrent *otitis media* (middle ear infection) in children often requires placement of tubes in the tympanic membrane that facilitate drainage of fluid from the ear. Removal of these tubes after treatment results in holes in the tympanic membrane that do not heal in a significant fraction of cases (10-20%). Overall, the number of patients who require tympanostom tubes estimated at 2,000,000 patients per year (Isaacson and Rosenfeld, *Ped. Otolaryngol.*, 43:1183, 1996). Of these, it is estimated that 3.5-10% (70,000-200,000 patients/year) will develop persistent tympanic perforations requiring patch treatments (Golz et al., *Otolaryngol.*, 120:524, 1999).

The standard procedure for filling such perforations involves sculpting auricular cartilage harvested from the patient to fit into the tympanic membrane defect. This sculpting procedure is time consuming, inexact, and difficult to reproduce.

Tissue engineering involves the regeneration of tissues such as bone and cartilage by seeding cells onto a customized biodegradable polymer scaffold to provide a three dimensional environment that promotes matrix production. This structure anchors cells and permits nutrition and gas exchange with the ultimate formation of new tissue in the shape of the polymer material. See, e.g., Vacanti et al., 1994, *Transplant. Proc.*, 26:3309-3310; and Puelacher et al., 1994, *Biomaterials*, 15:774-778.

## SUMMARY

The invention is based on the discovery that industrial design and manufacturing techniques, such as injection molding, can be used to create detailed, three-dimensional constructs for patching holes in the tympanic membrane, the eardrum. These constructs are made of, e.g., living cartilage and fibroblasts. The new methods involve the use of tissue engineering technology to generate precisely shaped implants or constructs to fill the perforations using scaffold molding and cell/polymer injection molding techniques.

In general, the invention features methods of making a living tissue construct for repairing a perforation in a tympanic membrane by providing a negative mold having a defined, e.g., predetermined, negative shape of the construct; suspending isolated tissue precursor cells in a hydrogel to form a liquid hydrogel-precursor cell composition; introducing the liquid hydrogel-precursor cell composition into the mold; inducing gel formation to solidify the liquid hydrogel-precursor cell composition to form a living tissue construct; and removing the living tissue construct from the mold after gel formation, wherein the construct has a shape suitable for repairing a perforation in a tympanic membrane.

In these methods, the tissue precursor cells can be chondrocytes or fibroblasts, or a combination thereof, and the hydrogel can be alginate, chitosan, pluronic, collagen, or agarose. If the hydrogel is alginate, the concentration can be from 0.5% to 8%, e.g., from 1% to 4%, e.g., approximately 2%. The gel formation can be induced by contacting the liquid hydrogel with a suitable concentration of a divalent cation, such as  $\text{Ca}^{++}$ , e.g., at a concentration of about 0.2 mg/ml of alginate solution. The tissue precursor cells can be cultured in the solidified hydrogel construct, e.g., *in vitro*, for a period of 1 to 30 days prior to implantation. In these methods, the negative mold can be prepared using CAD/CAM or rapid prototyping.

In another aspect, the invention features a method of repairing a perforation in a tympanic membrane in a mammal by providing a suitable negative mold having a negative shape of the living tissue construct; suspending isolated tissue precursor cells in a hydrogel to form a liquid hydrogel-precursor cell composition; introducing the liquid hydrogel-precursor cell composition into the mold; inducing gel formation to solidify the liquid hydrogel-precursor cell composition to form a living tissue construct; removing the tissue construct

from the mold after gel formation; and implanting the tissue construct into the perforation in the tympanic membrane in the mammal.

An alternative method of repairing a perforation in a tympanic membrane in a mammal includes obtaining a living tissue construct shaped to fit into the perforation; and  
 5 implanting the tissue construct into the perforation in the tympanic membrane in the mammal. In this method, the construct can be prepared by the methods described herein.

The invention also features an injection-molded living tissue repair construct made by the methods described herein. In these methods and constructs, the hydrogels can be polysaccharides, proteins, polyphosphazenes, poly(oxy-ethylene)-poly(oxypropylene) block  
 10 polymers, poly(oxyethylene)-poly(oxypropylene) block polymers of ethylene diamine, poly(acrylic acids), poly(methacrylic acids), copolymers of acrylic acid and methacrylic acid, poly(vinyl acetate), and sulfonated polymers.

A “hydrogel” is a substance formed when an organic polymer (natural or synthetic) is set or solidified to create a three-dimensional open-lattice structure that entraps molecules of  
 15 water or other solution to form a gel. The solidification can occur, e.g., by aggregation, coagulation, hydrophobic interactions, or cross-linking. The hydrogels employed in this invention rapidly solidify to keep the cells evenly suspended within a mold until the gel solidifies. The hydrogels are also biocompatible, e.g., not toxic, to cells suspended in the hydrogel.

A “hydrogel-cell composition” is a suspension of a hydrogel containing desired tissue precursor cells. These cells can be isolated directly from a tissue source or can be obtained  
 20 from a cell culture. A “tissue” is a collection or aggregation of particular cells embedded within its natural matrix, wherein the natural matrix is produced by the particular living cells. A “living tissue construct” is a collection of living cells that have a defined shape and structure. To be “living,” the cells must at least have a capacity for metabolism, but need not  
 25 be able to grow or reproduce in all embodiments. Of course, a living tissue construct can also include, and in some embodiments preferably includes, cells that grow and/or reproduce.

“Tissue precursor cells” are cells that form the basis of new tissue. Tissue cells can be “organ cells,” which include hepatocytes, islet cells, cells of intestinal origin, muscle cells,  
 30 heart cells, cartilage cells, bone cells, kidney cells, cells of hair follicles, cells from the vitreous humor in the eyes, cells from the brain, and other cells acting primarily to synthesize

and secret, or to metabolize materials. In some embodiments, these cells can be fully mature and differentiated cells. In addition, tissue precursor cells can be so-called "stem" cells or "progenitor" cells that are partially differentiated or undifferentiated precursor cells that can form a number of different types of specific cells under different ambient conditions, and that multiply and/or differentiate to form a new tissue.

An "isolated" tissue precursor cell, such as an isolated nerve cell, or an isolated nerve stem or progenitor cell or bone cell, or bone stem or progenitor cell, is a cell that has been removed from its natural environment in a tissue within an animal, and cultured in vitro, at least temporarily. The term covers single isolated cells, as well as cultures of "isolated" stem cells, that have been significantly enriched for the stem or progenitor cells with few or no differentiated cells.

As used herein, "negative mold" means a concave mold into which a liquid can be introduced for subsequent solidification. The mold is "negative" in the sense that concavity of the mold represents convexity in the object to be formed.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, useful methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflicting subject matter, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The invention has many advantages. For example, the new methods reduce the number of manufacturing steps needed to prepare precise, three-dimensional eardrum repair constructs. The new methods also provide increased uniformity of cell seeding throughout the construct, and increased efficiency of cell containment within the construct.

Additional advantages include: 1) elimination of variability in repair construct ("plug") geometry due to surgical skill; 2) decrease in interoperative time by elimination of harvest and sculpting steps; 3) availability of an off-the-shelf component, which will allow for choice of variously sized implants during surgery; and 4) the fabrication of custom-designed implants via injection-molding technology.

The new technology also has significant advantages over the development of synthetic prosthesis to fill these defects. Since these patches must remain in place permanently for long-term efficacy, synthetic implants are less desirable due to the possibility of chronic inflammation from foreign body response. Placing engineered tissue constructs, rather than synthetic patches, into the defect decreases the likelihood of immune response.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram of the injection molding process. Bovine articular cartilage was digested in collagenase II (3 mg/ml) at 37°C for 12-18 hours. Chondrocytes were concentrated to 1, 2.5, and 5 x 10<sup>7</sup> cells/ml and suspended in a solution of 2% alginate. Immediately before injection into the mold, sterilized CaSO<sub>4</sub> (0.2 gm/ml of alginate) in PBS was mixed with chondrocytes in alginate to initiate gel formation. The chondrocyte/alginate/CaSO<sub>4</sub> mixture was injected to the sterilized mold using a syringe and needle. Formed shapes were removed from molds 15 minutes after injection.

FIG. 2 is a schematic diagram of a tympanic membrane repair construct positive model that is used to prepare a negative mold. Such a model can be a computer image, or a three-dimensional, physical model.

### DETAILED DESCRIPTION

The invention utilizes tissue-engineering techniques to generate new living tissue constructs or implants that are used to patch holes in tympanic membranes. In contrast to conventional tissue engineering techniques, that involve creating a shaped scaffold and then seeding the shaped scaffold with cells in a separate step, the invention utilizes a suspension of cells in a solution from which a hydrogel is formed at a controlled gelation rate. Specifically, negative molds of implants used to fill perforations in the tympanic membrane are produced either by starting with a positive mold or a custom-designed drawing via computer aided design (CAD) (Fig. 2). Thereafter, standard molding materials and software are used to make negative molds from three-dimensional images or positive models. The

new methods enable the formation of a variety of negative molds to vary the size and shape of the patch to fit a given patient.

The new methods can be used to grow new eardrum tissue by using a hydrogel-cell composition that is formed into a precise shape using new injection molding techniques. To guide the development and shape of the new tissue, a precise negative mold is created, and the hydrogel-cell composition is delivered into the mold and cured to form a solid, three-dimensional living tissue construct, which is implanted into a hole in the patient's eardrum after the hydrogel-cell composition is solidified. The construct can be first placed into an *in vitro* controlled environment to allow the cells to grow for a period of days or weeks within the solidified hydrogel, or the construct can be implanted directly after solidification. In the following subsections, suitable molding techniques, hydrogels, cells, and delivery methods will be described, along with illustrative examples.

#### General Methodology

As with any process based on injection molding, the size and shape of the shaped product is determined by the size and shape of the negative mold. Thus, the invention can be employed to produce an eardrum implant or construct having essentially any size and shape, with the size and shape being precisely controlled. The living tissue construct can be used for the repair of perforations in the tympanic membrane.

Because injection molding allows for the use of a precise negative mold, detailed three-dimensional structural information from computer-aided drafting (CAD) can be used together with computer-aided manufacturing (CAM) and rapid prototyping to produce high quality molds in which the eardrum tissue constructs are formed. CAD/CAM hardware and software are commercially available and can be employed using techniques known in the art to design and produce molds suitable for use in the invention.

Although CAD/CAM techniques can be used in the design and production of molds they are not required. In some embodiments of the invention, a mold is constructed manually, e.g., by using a Silastic ERTV mold making kit (Dow Corning). For example, negative molds can be fabricated by immersing half of a positive model in a bed formed from the mixed components of an ERTV kit. This mixture is then placed in an 80° F oven for 30 minutes. After the bottom is hardened, approximately the same amount of uncured silastic is

poured on top to a height of 2 cm. This is again cured at 80° F for 30 minutes. After separation of the top and lower sets of the mold, the model is removed.

As shown in FIG. 1, cells are extracted from a source, such as cartilage, using standard techniques. For example, cartilage can be cut into small pieces of 1 to 3 mm<sup>3</sup>, and then disrupted with an enzyme or other chemical that separates the cells but does not destroy them. For example, collagenase works well for disrupting collagen into separate cells. Fibroblasts can be isolated from skin by a similar method. For example, the dermis can be separated from the skin and minced, and then treated with collagenase to disrupt the dermis into separate cells, which are mostly fibroblasts. In both cases, the cells are filtered to remove extracellular matrix debris, and are centrifuged and resuspended.

A combination of fibroblasts and chondrocytes is then suspended in a hydrogel, such as a diluted alginate solution (e.g., 0.1-3%), to produce a hydrogel-cell composition that can be delivered into the mold in liquid form, and is then injection molded into a pre-constructed negative mold. The hydrogel-cell composition is introduced into the mold simultaneously with a precise curing composition, such as 0.2 g/ml CaSO<sub>4</sub>. After a predetermined time, such as 15 minutes for alginate, the hydrogel-cell composition is removed from the mold after it has solidified or cured.

The molded eardrum construct can then be implanted directly into the patient's eardrum or it can be cultured *in vitro* for a time sufficient for tissue to develop.

### Hydrogels

Any suitable polymer hydrogel can be used in methods of the invention. A suitable polymer hydrogel is one that is biologically compatible, non-cytotoxic, and formed through controllable crosslinking (gelation), under conditions compatible with viability of isolated cells suspended in the solution that undergoes gelation. Various polymer hydrogels meeting these requirements are known in the art and can be used in the practice of the invention. Examples of different hydrogels suitable for practicing this invention, include, but are not limited to: (1) hydrogels cross-linked by ions, e.g., sodium alginate; (2) temperature dependent hydrogels that solidify or set at body temperature, e.g., PLURONICS<sup>TM</sup>; (3) hydrogels set by exposure to either visible or ultraviolet light, e.g., polyethylene glycol



polylactic acid copolymers with acrylate end groups; and (4) hydrogels that are set or solidified upon a change in pH, e.g., TETRONICS™.

Examples of materials that can be used to form these different hydrogels include polysaccharides such as alginate, polyphosphazenes, and polyacrylates, which are cross-linked ionically, or block copolymers such as PLURONICS™ (also known as POLOXAMERS™), which are poly(oxyethylene)-poly(oxypropylene) block polymers solidified by changes in temperature, or TETRONICS™ (also known as POLOXAMINES™), which are poly(oxyethylene)-poly(oxypropylene) block polymers of ethylene diamine solidified by changes in pH.

#### Ionic Hydrogels

Ionic polysaccharides, such as alginates and chitosan, can be used to suspend living cells. Tissue precursor cells are mixed with a polysaccharide solution, the solution is delivered into a mold, and then solidifies when the proper concentrations of ions are added. For example, alginate is an anionic polysaccharide capable of reversible gelation in the presence of an effective concentration of a divalent cation. A hydrogel can be produced by cross-linking the anionic salt of alginic acid, a carbohydrate polymer isolated from seaweed, with ions, such as calcium cations. The strength of the hydrogel increases with either increasing concentrations of calcium ions or alginate. For example, U.S. Patent No. 4,352,883 describes the ionic cross-linking of alginate with divalent cations, in water, at room temperature, to form a hydrogel matrix.

In a more specific example,  $\text{Ca}^{++}$  can be supplied conveniently in the form of  $\text{CaSO}_4$ . In some embodiments of the invention,  $\text{CaSO}_4$  is added in the amount of 0.1 to 0.5 gram, e.g., approximately 0.2 gram, per milliliter of a 2% solution of alginate. If the amount of soluble alginate is increased or decreased, the amount of divalent cation may need to be adjusted accordingly. Such adjustment is within ordinary skill in the art. The solubility of  $\text{CaSO}_4$  is 0.209 g/ml, which is much lower than that of  $\text{CaCl}_2$  (74.5 g/ml), which is the crosslinking agent typically used in for encapsulation of cells in alginate. See Beekman et al., 1997, Exper. Cell Res., 237:135-141. At a concentration of  $\text{CaSO}_4$  near or above the solubility limit,  $\text{Ca}^{2+}$  in solution begins to crosslink alginate, and it is replenished by solubilization of precipitated  $\text{CaSO}_4$ . This results in a significant slowing of the crosslinking process. Such slowing can be advantageous, because it allows the alginate/ $\text{CaSO}_4$  mixture to

be injected into a mold before the completion of the crosslinking process occurs in the shaped implant.

In general, these polymers are at least partially soluble in aqueous solutions, e.g., water, or aqueous alcohol solutions that have charged side groups, or a monovalent ionic salt thereof. There are many examples of polymers with acidic side groups that can be reacted with cations, e.g., poly(phosphazenes), poly(acrylic acids), and poly(methacrylic acids). Examples of acidic groups include carboxylic acid groups, sulfonic acid groups, and halogenated (preferably fluorinated) alcohol groups. Examples of polymers with basic side groups that can react with anions are poly(vinyl amines), poly(vinyl pyridine), and poly(vinyl imidazole).

Polyphosphazenes are polymers with backbones consisting of nitrogen and phosphorous atoms separated by alternating single and double bonds. Each phosphorous atom is covalently bonded to two side chains. Polyphosphazenes that can be used have a majority of side chains that are acidic and capable of forming salt bridges with di- or trivalent cations. Examples of acidic side chains are carboxylic acid groups and sulfonic acid groups.

Bioerodible polyphosphazenes have at least two differing types of side chains, acidic side groups capable of forming salt bridges with multivalent cations, and side groups that hydrolyze under in vivo conditions, e.g., imidazole groups, amino acid esters, glycerol, and glucosyl. Bioerodible or biodegradable polymers, i.e., polymers that dissolve or degrade within a period that is acceptable in the desired application (usually in vivo therapy), will degrade in less than about five years and most preferably in less than about one year, once exposed to a physiological solution of pH 6-8 having a temperature of between about 25°C and 38°C. Hydrolysis of the side chain results in erosion of the polymer. Examples of hydrolyzing side chains are unsubstituted and substituted imidizoles and amino acid esters in which the side chain is bonded to the phosphorous atom through an amino linkage.

Methods for synthesis and the analysis of various types of polyphosphazenes are described in U.S. Patent Nos. 4,440,921, 4,495,174, and 4,880,622. Methods for the synthesis of the other polymers described above are known to those skilled in the art. See, for example Concise Encyclopedia of Polymer Science and Engineering, J.I. Kroschwitz, editor (John Wiley and Sons, New York, NY, 1990). Many polymers, such as poly(acrylic acid), alginates, and PLURONICS™, are commercially available.

Water soluble polymers with charged side groups are cross-linked by reacting the polymer with an aqueous solution containing multivalent ions of the opposite charge, either multivalent cations if the polymer has acidic side groups, or multivalent anions if the polymer has basic side groups. Cations for cross-linking the polymers with acidic side groups to form a hydrogel include divalent and trivalent cations such as copper, calcium, aluminum, magnesium, and strontium. Aqueous solutions of the salts of these cations are added to the polymers to form soft, highly swollen hydrogels.

Anions for cross-linking the polymers to form a hydrogel include divalent and trivalent anions such as low molecular weight dicarboxylate ions, terephthalate ions, sulfate ions, and carbonate ions. Aqueous solutions of the salts of these anions are added to the polymers to form soft, highly swollen hydrogels, as described with respect to cations.

For purposes of preventing the passage of antibodies into the hydrogel, but allowing the entry of nutrients, a useful polymer size in the hydrogel is in the range of between 10,000 D and 18,500 D. Smaller polymers result in gels of higher density with smaller pores.

#### Temperature-Dependent Hydrogels

Temperature-dependent, or thermosensitive, hydrogels can be use in the methods of the invention. These hydrogels have so-called “reverse gelation” properties, i.e., they are liquids at or below room temperature, and gel when warmed to higher temperatures, e.g., at or above body temperature. Thus, these hydrogels can be easily injected into a mold at or below room temperature as a liquid and automatically form a semi-solid gel when warmed to or above body temperature. Examples of such temperature-dependent hydrogels are PLURONICS™ (BASF-Wyandotte), such as polyoxyethylene-polyoxypropylene F-108, F-68, and F-127, poly (N-isopropylacrylamide), and N-isopropylacrylamide copolymers.

These copolymers can be manipulated by standard techniques to affect their physical properties such as porosity, rate of degradation, transition temperature, and degree of rigidity. For example, the addition of low molecular weight saccharides in the presence and absence of salts affects the lower critical solution temperature (LCST) of typical thermosensitive polymers. In addition, when these gels are prepared at concentrations ranging between 5 and 25% (W/V) by dispersion at 4°C, the viscosity and the gel-sol transition temperature are affected, the gel-sol transition temperature being inversely related to the concentration.

These gels have diffusion characteristics capable of allowing cells to survive and be nourished.

U.S. Pat. No. 4,188,373 describes using PLURONIC<sup>TM</sup> polyols in aqueous compositions to provide thermal gelling aqueous systems. U.S. Pat. Nos. 4,474,751, '752, '753, and 4,478,822 describe drug delivery systems which utilize thermosetting polyoxyalkylene gels; with these systems, both the gel transition temperature and/or the rigidity of the gel can be modified by adjustment of the pH and/or the ionic strength, as well as by the concentration of the polymer.

#### pH-Dependent Hydrogels

Other hydrogels suitable for use in the methods of the invention are pH-dependent. These hydrogels are liquids at, below, or above specific pH values, and gel when exposed to specific pHs, e.g., 7.35 to 7.45, the normal pH range of extracellular fluids within the human body. Thus, these hydrogels can be easily delivered into a mold as a liquid and form a semi-solid gel when exposed to the proper pH. Examples of such pH-dependent hydrogels are TETRONICS<sup>TM</sup> (BASF-Wyandotte) polyoxyethylene-polyoxypropylene polymers of ethylene diamine, poly(diethyl aminoethyl methacrylate-g-ethylene glycol), and poly(2-hydroxymethyl methacrylate). These copolymers can be manipulated by standard techniques to affect their physical properties.

An example of another a useful pH-dependent hydrogel is collagen. Collagen is a protein that undergoes cross-linking in response to shift in pH from alkaline to acid, e.g., a shift from a pH in the range of < 2 to a pH in the range of > 6. See, e.g., Bell et al., 1979, Proc. Nat. Acad. Sci., 76:1274.

#### Light Solidified Hydrogels

Other hydrogels that can be used in the methods of the invention are solidified by either visible or ultraviolet light. These hydrogels are made of macromers including a water-soluble region, a biodegradable region, and at least two polymerizable regions as described in U.S. Patent No. 5,410,016. For example, the hydrogel can begin with a biodegradable, polymerizable macromer including a core, an extension on each end of the core, and an end cap on each extension. The core is a hydrophilic polymer, the extensions are biodegradable polymers, and the end caps are oligomers capable of cross-linking the macromers upon exposure to visible or ultraviolet light, e.g., long wavelength ultraviolet light. These types of

hydrogels can be used with transparent or translucent molds, or with molds that have optic fibers that introduce light into the mold.

Examples of such light solidified hydrogels include polyethylene oxide block copolymers, polyethylene glycol polylactic acid copolymers with acrylate end groups, and 10K polyethylene glycol-glycolide copolymer capped by an acrylate at both ends. As with the PLURONIC™ hydrogels, the copolymers comprising these hydrogels can be manipulated by standard techniques to modify their physical properties such as rate of degradation, differences in crystallinity, and degree of rigidity.

#### Tissue Precursor Cells

Various types of isolated cells or tissue precursor cells (e.g., progenitor or stem cells) can be used in methods according to the invention. However, isolated chondrocytes and fibroblasts are preferred to create patches for the eardrum.

Tissue precursor cells can be obtained directly from a mammalian donor, e.g., a patient's own cells, from a culture of cells from a donor, or from established cell culture lines. Preferably the mammal is a mouse, rat, rabbit, guinea pig, hamster, cow, pig, horse, goat, sheep, dog, cat, and most preferably, the mammal is a human. Cells of the same species and preferably of the same immunological profile can be obtained by biopsy, either from the patient or a close relative. Using standard cell culture techniques and conditions, the cells are then grown in culture until confluent and used when needed. The cells are preferably cultured only until a sufficient number of cells have been obtained for a particular application.

If cells are used that may elicit an immune reaction, such as human fibroblast cells from an immunologically distinct donor, then the recipient can be immunosuppressed as needed, for example, using a schedule of steroids and other immunosuppressant drugs such as cyclosporine. However, the use of autologous cells will avoid such an immunologic reaction.

Cells can be obtained directly from a donor, washed, suspended in a selected hydrogel before being injected into a mold. To enhance cell growth, the cells are added or mixed with the hydrogel just prior to injection.

Cells obtained by biopsy are harvested, cultured, and then passaged as necessary to remove contaminating, unwanted cells. The isolation of chondrocytes is described in the examples below. Fibroblasts and other cells can be isolated in a similar fashion.

Cell viability can be assessed using standard techniques including visual observation with a light or scanning electron microscope, histology, or quantitative assessment with radioisotopes. The biological function or metabolism of the cells can be determined using a combination of the above techniques and standard functional assays.

Examples of cells that can be delivered into molds and subsequently grow new tissue in living tissue constructs include epidermal cells; chondrocytes and other cells that form cartilage ("cartilage-forming cells"); dermal cells; fibroblasts; epithelial cells; endothelial cells; ear canal cells; and cells derived from the tympanic membrane.

#### Preparation of Hydrogel-Cell Compositions

First, a hydrogel of choice is prepared using standard techniques. For example, a biodegradable, thermosensitive polymer at a concentration ranging between 5 and 25% (W/V) is useful for the present invention. If the hydrogel is an alginate, it can be dissolved in an aqueous solution, for example, a 0.1 M potassium phosphate solution, at physiological pH, to a concentration between 0.1 to 4% by weight, e.g., 2%, to form an ionic hydrogel.

Second, isolated tissue precursor cells are suspended in the polymer solution at a concentration mimicking that of the tissue to be generated. The optimal concentration of cells to be delivered into the mold is determined on a case by case basis, and may vary depending on cellular type and the region of the patient's body into which the living tissue implant is inserted. Optimization experiments require modifying only a few parameters, i.e., the cell concentration or the hydrogel concentration, to provide optimal viscosity and cell number to support the growth of new tissue. For chondrocytes, the cell concentration range is from about 10 million cells/ml to about 100 million cells/ml, e.g., from about 25 million cells/ml to about 50 million cells/ml.

### Implantation of Living Eardrum Tissue Constructs

To implant a living eardrum tissue construct, the perforation in the patient's eardrum is cleared of any dead cells or tissue, and the construct is implanted directly into the perforation using standard techniques.

Cartilage is a specialized type of dense connective tissue consisting of cells embedded in a matrix. There are several kinds of cartilage, and any one of these can be used in the new methods. Hyaline cartilage is a bluish-white, glassy translucent cartilage having a homogeneous matrix containing collagenous fibers that is found in articular cartilage, in costal cartilages, in the septum of the nose, and in the larynx and trachea. Articular cartilage is hyaline cartilage covering the articular surfaces of bones. Costal cartilage connects the true ribs and the sternum. Fibrous cartilage contains collagen fibers. Yellow cartilage is a network of elastic fibers holding cartilage cells which is found primarily in the epiglottis, the external ear, and the auditory tube. By harvesting the appropriate chondrocyte precursor cells, any of these types of cartilage tissue can be grown using the methods of the invention.

Over time, e.g., over a period of approximately six weeks, the eardrum construct will become vascularized and the chondrocytes will grow new cartilaginous tissue that takes the shape of the eardrum patch and engrafts to existing tympanic membrane tissue.

### **EXAMPLES**

#### **Example 1 - Isolation of Chondrocytes**

Freshly slaughtered calf forelimbs were obtained from a local slaughterhouse within 6 hours of sacrifice. The forelimbs were dissected under sterile conditions to expose the articular surfaces of the glenohumeral and humeroulnar joint. Cartilage fragments were sharply curetted off the articular surface of each joint, were subjected to collagenase II digestion (3 mg/ml) (Worthington Biochemical Corp, freehold, NJ USA.) at 37°C for 12 to 18 hours. Preparation of chondrocytes was in accordance with methods described in Klagsburn, 1979, Meth. Enzymol., 58:560-564.

The resulting cell suspension was passed through a sterile 250  $\mu$  polypropylene mesh filter (Spectra/Mesh 146-426 Spectrum Medical Industries, Inc., Laguna Hills, CA, and USA.). The filtrate was centrifuged at 6000 rpm, and the resulting cell pellet was washed twice with copious amounts of Dulbecco phosphate buffered-saline (PBS) (Gibco,

Grand Island, NY, USA) without  $\text{Ca}^{2+}$ . Cell number was determined using a hemocytometer and the cell viability determined using trypan blue dye (Sigma-Aldrich, Irvine, KA, USA.). Chondrocyte suspensions were concentrated to various cellular densities of 10, 25, and  $50 \times 10^6$  cells/ml and suspended in a solution of 2% alginate.

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### Example 2 - Construction of Molds

A three-dimensional reconstruction of a positive template for a tissue-engineered patch for a tympanic perforation is generated by computer-aided design (CAD) using standard techniques. Fig. 2 illustrates the virtual template. This image is ported directly to software in a mold-making device, which generates a negative mold.

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### Example 3 - Alginate Construct Formation

Isolated fibroblast and cartilage cells are resuspended in a 2% sterile sodium alginate (Pronova Biopolymer, Norway) solution (0.1M  $\text{K}_2\text{HPO}_4$ , 0.135M NaCl, pH 7.4), which has previously been sterilized with a 0.45  $\mu\text{m}$  filter to yield various cellular concentrations of 10, 25, and  $50 \times 10^6$ /ml alginate solution. Immediately before injection into the silicon mold, sterilized  $\text{CaSO}_4$  (0.2 gm/ml of alginate) in PBS solution is mixed with chondrocyte-alginate construct to initiate gel formation. The chondrocyte-fibroblast/alginate/ $\text{CaSO}_4$  mixture is delivered into the sterilized mold of Example 2 using a 10 ml syringe and an 18.5 gauge needle. Formed shapes are removed from molds 10 minutes after injection. FIG. 1 illustrates the overall method.

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The solidified construct can be put into culture under standard conditions, e.g., for one week, to allow the cells to grow to confluence within the hydrogel construct. Alternatively, the construct can be implanted directly into a patient.

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## OTHER EMBODIMENTS

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention.

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